Journal of Cell Science 109, 2649-2660 (1996)
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JCS1245

A maternal requirement for glutamine synthetase I for the mitotic cycles of syncytial *Drosophila* embryos

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SUMMARY

We describe the maternal effect phenotype of a hypomorphic mutation in the *Drosophila* gene for glutamine synthetase I (GSI). The extent of development of embryos derived from homozygous mutant females is variable, although most mutant embryos fail to survive past germband elongation and none develop into larvae. These embryos are characterised by an increase in the number of yolk-like nuclei following nuclear migration to the cortex. These nuclei appear to fall into the interior of the embryo from the cortex at blastoderm. As they do so, the majority continue to show association with PCNA in synchrony with nuclei at the cortex, suggesting some continuity of the synchrony of DNA replication. However, the occurrence of nuclei that have lost cell cycle synchrony with their neighbours is not uncommon. Immunostaining of mutant embryos revealed a range of mitotic defects, ultimately resulting in nuclear fusion events, division failure or other mitotic abnormalities. A high proportion of these mitotic figures show chromatin bridging at anaphase and telophase consistent with progression through mitosis in the presence of incompletely replicated DNA. GSI is responsible for the ATP-dependent amination of glutamate to produce glutamine, which is required in the formation of amino acids, purines and pyrimidines. We discuss how the loss of glutamine could depress both protein and DNA synthesis and lead to a variety of mitotic defects in this embryonic system that lacks certain checkpoint controls.

Key words: *Drosophila*, Cell cycle, Mitosis, Glutamine synthetase

INTRODUCTION

The *Drosophila melanogaster* embryo is initially a syncytium in which there are 13 parasynchronous nuclear divisions following fertilisation. The maternal supply of cell cycle components permits rapid division cycles lasting only 10 minutes, in which there are no G_1 or G_2 phases. The first mitotic division takes place in the interior of the embryo, towards the anterior end, clustered around the site of pronuclear fusion. During each of the following divisions the nuclei move away from each other, then during telophase of cycles 8 and 9 they migrate out to the cortex of the embryo (Zalokar and Erk, 1976). Nuclei at the posterior end of the embryo, destined to become incorporated into the pole cells arrive at the cortex first, during cycle 9, ahead of the somatic nuclei which reach the cortex at interphase of cycle 10. Drug inhibition studies indicate that microtubules are required for this cortical migration (Zalokar and Erk, 1976). Once the nuclei approach the cortex, new forces come into play to keep them there. Foe et al. (1993) propose that these forces rely on the interaction of the astral microtubules with yolk particles in the interior of the embryo. These astral microtubules, nucleated by the centrosomes associated with each nucleus, appear to repel yolk particles, resulting in a region of yolk free cytoplasm at the surface of the embryo. The force on the yolk particles is applied from both sides of the embryo, so that they are maintained in the centre of the embryo, whilst the nuclei attached to the centrosomes are pushed against the surface. Once at the surface, a microtubule based repulsion force between adjacent asters, similar to that involved in cortical migration, may maintain the equidistant separation of nuclei in the plane of the plasma membrane (Zalokar and Erk, 1976; Hatanaka and Okada, 1991), although an interaction of astral microtubules with cortical actin also contributes to the organisation of these surface nuclei (Sullivan et al., 1993a).

As the majority of nuclei migrate towards the cortex, some nuclei remain in the centre of the embryo. These are the yolk nuclei, which continue to cycle in synchrony with the surface nuclei until cycle 10, when they lag behind the syncytial blastoderm nuclei by a few minutes and then begin endoreplication to become polyploid. The yolk nuclei lose their associated centrosomes as they begin endoreplication (Callaini and Dallai, 1991), an event which may be a prerequisite for uncoupling DNA replication from mitosis. The loss of their centrosomes also results in the loss of equidistant spacing, with adjacent yolk nuclei frequently fusing (Foe and Alberts, 1983). The basis of the regulation of these cycles is unknown, although it is apparent that the normal controls which prevent reinitiation of replication within a single cycle are somehow modified.

During the syncytial cycles, the usual dependency of mitosis on the completion of DNA replication is absent, and embryos treated with aphidicolin to inhibit DNA synthesis, undergo repeated cycles of centrosome replication, lamina

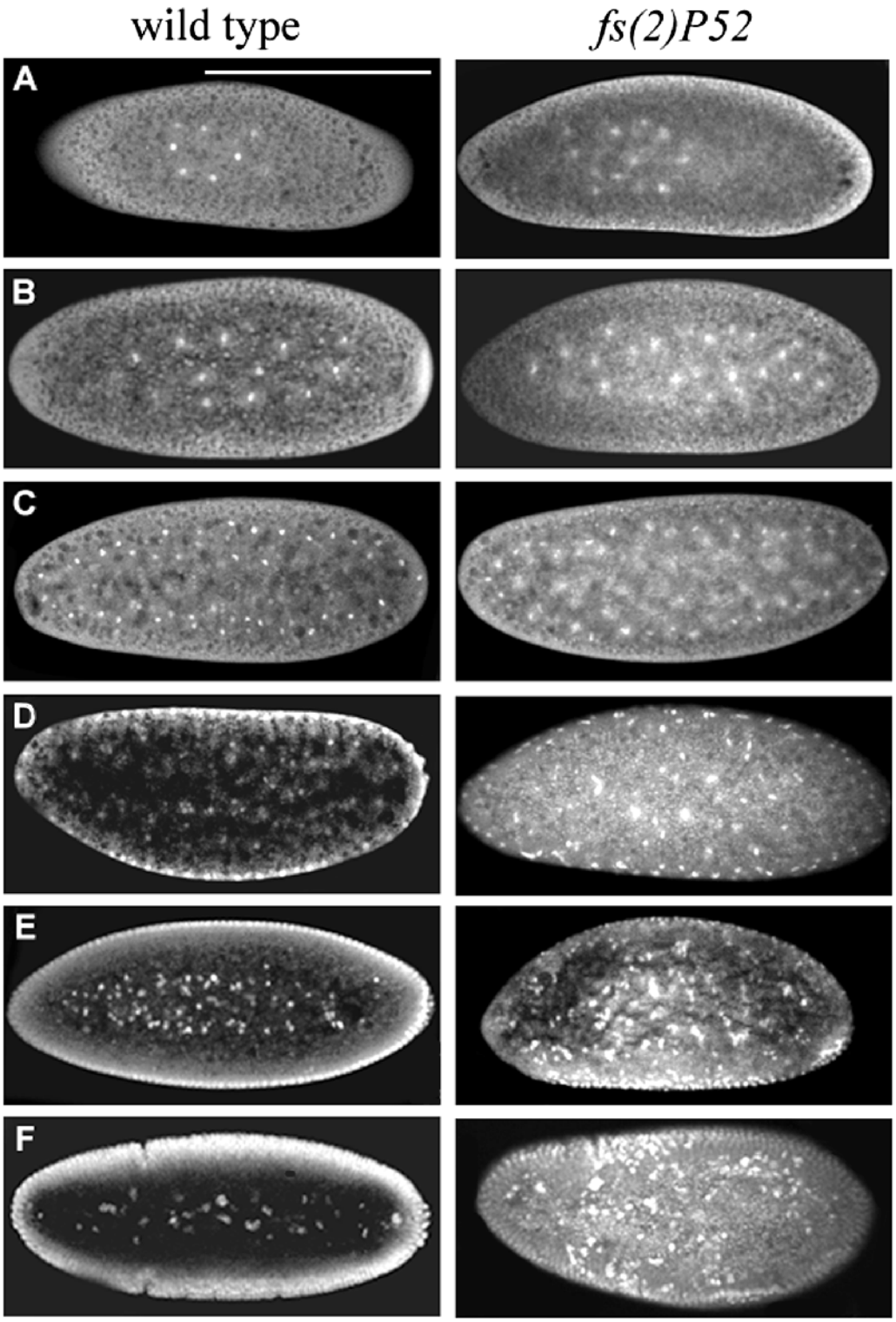

Fig. 1. *fs(2)P52* embryos have an increased number of polyploid, pseudo-yolk nuclei. Wild-type (left-hand panels) and *fs(2)P52* embryos (right-hand panels) were fixed, and their DNA was stained using propidium iodide. The figure illustrates the nuclear movements which occur as the embryos develop from cycle 4 (A) through to cycle 14 (F). In a preblastoderm wild-type embryo, the cluster of dividing nuclei originally located towards the anterior end of the embryo (A), expands into the posterior half of the egg during mitotic cycles 4 and 5 (B). After the first 7 divisions, which occur within the interior of the embryo, the nuclei begin to migrate to the periphery. Nuclei at the posterior end of the embryo reach the cortex ahead of the others, during cycle 9 (C). By the end of mitotic cycle 10, all of the nuclei are positioned at the surface of the embryo just beneath the plasma membrane (D), where they undergo 3 more synchronous divisions prior to cellularisation during the interphase of cycle 14 (E). The yolk nuclei seen in the interior of the syncytial blastoderm (E) originate from nuclei that failed to migrate to the cortex during nuclear cycle 8, together with those that fall from the surface between interphase 8 and 9. These yolk nuclei divide twice before undergoing two rounds of endoreplication. The embryos from females homozygous for the mutation *fs(2)P52*, appear normal during the early stages of their development (A-C). However, by cycle 10 the majority of mutant embryos have an increased number of yolk-like nuclei (D) and as development proceeds, the number of these nuclei increases dramatically (E-F). These internal nuclei apparent in the mutant embryos differ in their distribution from wild-type yolk nuclei, appearing throughout the interior of the embryo and not restricted to the small region along the anterior-posterior axis. Bar, 50 μm.

breakdown/re-assembly, and chromatin condensation/decondensation (Raff and Glover, 1988). Foe et al. (1993) postulate that a replication feedback mechanism would be an ineffective way of ensuring the co-ordination of events in a syncytial embryo, as a result of the proposed autocatalytic activity of cdc2-cyclin kinase. They propose that the leakage of an autocatalytic mitotic activator through the cytoplasm is responsible for propagating the mitotic wave, travelling through the nuclei of the syncytial blastoderm, which would not be stopped by a feedback signal from a single nucleus. Instead, any defective

nuclei are discarded into the centre of the syncytial embryo. Nuclei which fail to successfully replicate during S-phase are unable to complete anaphase and lose their centrosomes and associated anchorage at the cortex. In support of this, using time-lapse optical microscopy of living wild-type *Drosophila* embryos, Minden et al. (1989) noted that failed mitoses resulting from the incomplete separation of sister nuclei, resulted in irregularly shaped nuclei which eventually dropped out of the blastoderm monolayer into the yolky centre of the embryo. There are a number of mutants in which mitotic abnormalities lead to an increase in nuclei falling out of the cortex. One example is *daughterless-abo-like (dal)*, a maternal-effect semi-lethal mutation which causes incomplete centrosome separation, resulting in a non-functional mitotic spindle and nuclear division failure to produce irregularly shaped nuclei which fall into the interior of the embryo (Sullivan et al., 1990). Sullivan and co-workers (1993b) have suggested that in the *Drosophila* syncytial embryo the fidelity of nuclear divisions is maintained by a checkpoint which relies on division cycle delays to identify and discard products of division errors, rather than activating their repair as occurs in somatic cells.

In contrast, treatment of embryos with microtubule inhibitors such as colchicine, leads to a metaphase arrest, blocking all subsequent cycles of chromatin condensation/decondensation, nuclear envelope breakdown/reformation and DNA synthesis (Zalokar and Erk, 1976), indicating that there is a feedback or dependency coupling the centrosome/spindle cycles and the mitotic oscillator.

Mutations in many genes required for the nuclear divisions of early embryogenesis have been identified in screens for female sterility. The original mutant alleles of *polo* and *aurora*, for example, both result in a female sterile phenotype (Sunkel and Glover, 1988; Glover et al., 1995), although other alleles resulting in larval and pupal lethality, respectively, were subsequently isolated. Both *polo* and *aurora* encode Ser/Thr protein kinases, each of which is required for centrosome separation and the formation of a bipolar spindle (Llamazares, et al., 1991; Glover et al., 1995). We identified the mutant *fs(2)P52* in a screen of maternal effect mutants looking for defects in the nuclear division cycle of syncytial embryos. The single P-element insertion that causes this hypomorphic mutation has facilitated the cloning of the gene, which we have determined to encode glutamine synthetase I (GSI). In this paper we describe the embryonic phenotype resulting from the *fs(2)P52* mutation, and discuss possible roles for GSI in *Drosophila* embryogenesis.

MATERIALS AND METHODS

Immunofluorescent staining of embryos

Embryos were collected, dechorionated and fixed as described previously (Maldonada-Codina and Glover, 1992) except that 40% paraformaldehyde was used for fixation to permit the examination of microtubule distribution. Methanol devitillinisation, antibody staining and mounting were as previously described (Whitfield et al., 1990).

The polyclonal rabbit serum, Rb188 (against the centrosomal antigen CP190) (Whitfield et al., 1988) was used at a dilution of 1 in 500. The rat monoclonal YL1/2 (Seralabs) directed against tyrosinated α-tubulin was used at 1 in 10. For PCNA labelling, a rabbit polyclonal antibody against *Drosophila* PCNA (kind gift of Paul Fischer) was used at a 1 in 500 dilution. DNA was visualised using 1 mg/ml propidium iodide. Stained embryos were viewed using a Nikon Optiphot attached to a Bio-Rad MRC600 confocal microscope.

Reversion of *fs(2)P52*

To generate revertants of *fs(2)P52*, a marked chromosome carrying this mutation (*fs(2)P52 b pr px sp*) was exposed to a source of transposase, Δ2-3 (Robertson et al., 1988), by crossing *CyRoi* balanced females to *Sp/CyO; D2-3 Sb/TM6* males. The pUChsneo transposon can be mobilised in *fs(2)P52 b pr px sp/CyO; D2-3 Sb/+* females which are subsequently crossed to *Gla/SM1* males to generate stable lines of the potential revertants in which Δ2-3 is no longer present. Potential reversions are tested over the marked mutant chromosome

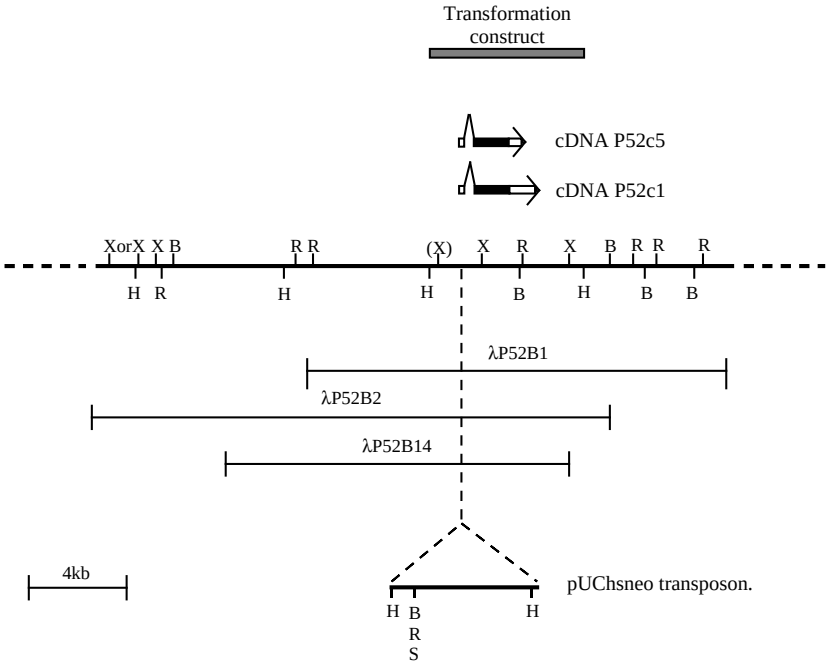

Fig. 2. Map of the cloned region from *fs(2)P52*. The genomic region in the vicinity of the pUChsneo insertion site of *fs(2)P52* was mapped using the following restriction endonucleases: *Xba*I (X), *Sal*I(S), *Bam*HI (B), *Hin*dIII (H) and *Eco*RI (R). The lambda DASH clones λP52B1, λP52B2 and λP52B14 were isolated from the genomic library using DNA flanking the transposon in *fs(2)P52* that was retrieved by plasmid rescue. The position and orientation of the pUChsneo transposon is shown relative to the genomic DNA and the corresponding clones. Inserts from the genomic clones identified 6 related clones from a 0-4 hour *Drosophila* embryonic cDNA library, which fell into 2 different size categories, the larger of which had an insert of 2.1 kb and is represented by P52c1, with the smaller clones having inserts of 1.5 kb, represented by P52c5. The open reading frames of these cDNAs are depicted as shaded regions. The figure also illustrates the 4.8 kb *Hin*dIII fragment used for germline transformation and rescue of the *fs(2)P52* mutant phenotype. This genomic region includes the *GsI* transcription unit, along with 0.5 kb of 5′ flanking DNA which includes all the necessary promoter sequence and 0.7 kb of 3′ flanking sequence.

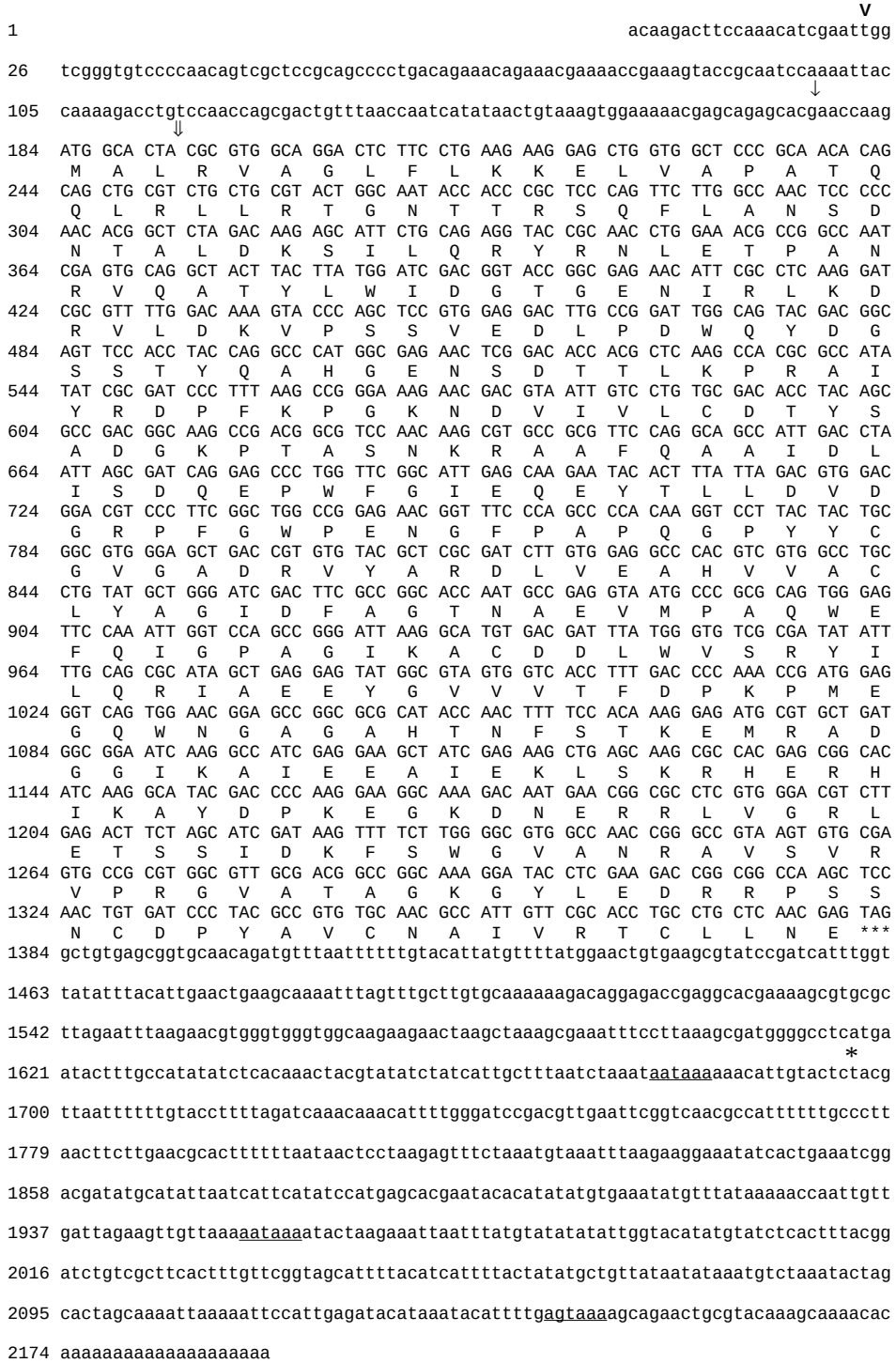

Fig. 3. Sequencing of the *throng* cDNA indicates that the cDNA encodes a protein identical to *D. melanogaster* glutamine synthetase I. DNA sequencing of the P52c1 clone insert showed it to encode a predicted protein of 399 amino acids with 100% homology to *Drosophila* glutamine synthetase I (GS I) (Caizzi et al., 1990). The positions of the intron (↓) and the *throng* pUChsneo insertion site (**v**) are indicated, as is the 3′ end of the smaller clones (*). The polyadenylation signals used by each of the 2 alternative transcripts are underlined. The construct pw8.P52^{m} has a mutagenised form of the *P52* gene, resulting from the insertion of 2 additional nucleotides in the coding region (⇓).

fs(2)P52 b pr px sp, with *trans*-heterozygous females able to produce viable progeny scoring as revertants.

To test whether the phenotypic reversion of *fs(2)P52* correlated with the loss of the pUChsneo transposon, a probe derived from a 5.3 kb genomic *Hin*dIII fragment spanning the insertion site was hybridised to a Southern blot of genomic DNA from 10 revertant lines. This probe recognises 2 bands of 7.0 kb and 3.8 kb in *Eco*RI digested genomic DNA from wild-type *Drosophila*, but recognises 3 bands of 6.4, 5.8 and 3.8 kb in *fs(2)P52* DNA digested with *Eco*RI, due to an *Eco*RI site located within the transposon. As predicted, the probe recognised only 2 bands in *Eco*RI digested DNA from 10 revertant lines, confirming that the reversion was the result of the excision of the pUChsneo transposon (data not shown). Although only 2 bands were detected in each of the revertant lines, the larger of the 2 was not always the predicted size of 7 kb, but was greater in 3 out of the 10 lines. This is probably the result of the imprecise excision of the pUChsneo transposon. In these 3 revertant lines we infer that some of the pUChsneo transposon has been left behind, yet it does not seem to interfere sufficiently with the adjacent gene to result in a female sterile phenotype.

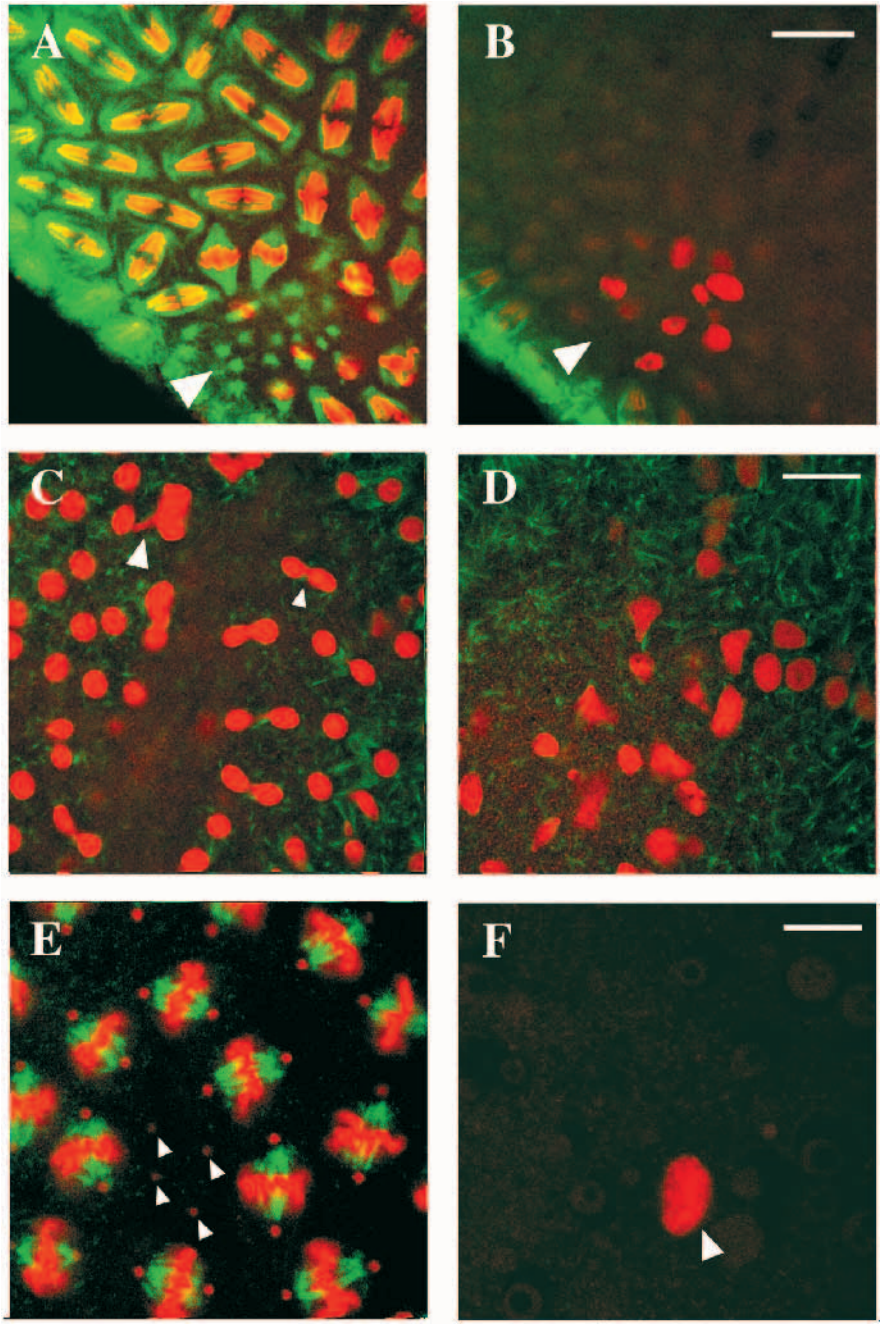

Fig. 4. Nuclei fall from the cortex of *GsI*^{fsP52} embryos leaving behind microtubular asters and centrosomes. *GsI*^{fsP52} embryos were fixed and incubated with YL1/2, a rat antibody which recognises tyrosinated α-tubulin, then with fluorescein-conjugated anti-rat IgG, and propidium iodide. The *GsI*^{fsP52} embryos illustrated in E and F were also incubated with Rb188, a rabbit polyclonal against the centrosome-associated protein, CP190, then with Texas red-conjugated anti-rabbit IgG (see Materials and Methods). In the merged images, DNA and CP190 are represented in red, with tubulin in green. In each of the 3 examples, the panels on the left are surface views of syncytial blastoderm embryos, while the panels on the right are equivalent views on a lower focal plane. In each case, nuclei which are missing from the cortex can be found in the corresponding position below the surface of the embryo. See text for details. Bars, 30 μm.

Molecular cloning by plasmid rescue

To recover genomic DNA flanking the pUChsneo transposon in *fs(2)P52*, DNA from *fs(2)P52* homozygotes was digested with *Bam*HI. This restriction endonuclease cuts at one end of the pUC8 sequence included in pUChsneo, as well as within the adjacent genomic DNA. The resulting DNA fragments were circularised using T4 DNA ligase and transformed into *E. coli*, where plasmids containing ampicillin resistance and the origin of replication conferred by the pUC8 sequence, were able to multiply and generate clones, when grown on LB under ampicillin selection. The flanking genomic DNA was used to probe a *Drosophila melanogaster* genomic library constructed using the lambda DASH vector (Stratagene). Three distinct clones were obtained from this screen (see Fig. 2) all of which hybridised in situ to 21B on the right arm of chromosome 2. Altogether, 50,000 plasmid clones from a 0-4 hour cDNA library (Brown and Kafatos, 1988) were screened using inserts from the genomic clones λP52B1 and λP52B2. Both probes recognised the same 2 related cDNA clones with inserts of 2.1 kb and 1.5 kb, respectively (see Fig. 2).

Sequence analysis

The larger of the 2 cDNAs, P52c1, was ligated into the *Not*I/*Hin*dIII sites of pBluescript SK$^+$ and pBluescript KS$^+$ (Stratagene). Working from an accurate restriction map, a set of specific restriction fragments of P52c1 cDNA were subcloned into pSK$^+$ and pKS$^+$. This enabled the sequencing of both strands of the cDNA using the M13 consensus primers of pSK$^+$ and pKS$^+$. The exact insertion site of the pUChsneo transposon in the *fs(2)P52* genome, was established by sequencing the original clone obtained from the plasmid rescue experiment. A specific set of sequencing primers based upon the known cDNA

sequence and the sequence of the pUChsneo transposon were designed for this purpose.

Double stranded DNA was prepared using the Wizard Magic DNA mini-preparation kit (Promega) and dideoxysequencing was conducted using the Sequenase 2.0 kit (Amersham) according to the manufacturer's instructions. Microgenie sequence analysis software (Beckman) was used to assemble accumulated sequences.

Germline transformation and rescue

This was performed essentially as described by Axton et al. (1990). Two constructs were separately microinjected into *w; Sb e ry D2-3/TM6* embryos (Robertson et al., 1988). The wild-type construct, pw8.P52 consists of a 4.8 kb *Hin*dIII fragment (see Fig. 2) inserted into the pw8 vector (Klemenz et al., 1987); 5 transformed lines were recovered from 38 fertile G_0 adults (13% transformation). The related construct, pw8.P52^m, has a mutagenised form of the *P52* gene. This was generated by cutting the plasmid at a unique *Mlu*I site 8 bp downstream of the start codon, end filling using ϕT4 DNA polymerase, then ligating the modified construct. This procedure resulted in the insertion of 2 additional base pairs into the *Mlu*I site, causing a frame shift and introducing a stop codon after the translation of only 10 inappropriate amino acids; 6 transformed lines were recovered from 28 fertile G_0 adults (21% transformation).

The ability of these constructs to rescue the female sterility of the *fs(2)P52* mutation was tested using lines in which the transforming transposon was carried on the third chromosome. Males from such lines (*w^a;P[w^+,P52$^{(m)}$] Sb e/TM6*) were crossed to *w^{1118}; fs(2)P52 b pr px sp/CyRoi* females. w^+ males carrying neither of the 2 balancer chromosomes, *TM6* or *CyRoi*, were again crossed to *w^{1118};fs(2)P52 b pr px sp/CyRoi* females to generate females homozygous for the *fs(2)P52* mutation and carrying the construct, *P[w^+,P52$^{(m)}$]*. Such females were tested for fertility by mating to Oregon-R males.

Administration of paraquat

Adult males, 0-48 hours of age, were exposed for 48 hours at 25°C, in vials (10 flies per vial) containing 2 Whatman GF/C 2.1 cm diameter filters, saturated with aqueous solutions of paraquat in 1% sucrose. The numbers of surviving flies, were scored immediately after treatment.

Exposure to X-irradiation

One hundred third instar larvae of undetermined sex were placed in a 5 cm diameter Petri dish and exposed to 2,000 rads of X-rays (520 rads/minute for 3.8 minutes). These were transferred to vials (20 larvae per vial) and survival was determined as the proportion of larvae successfully eclosing into adults. Survival after irradiation was compared to the survival of 100 unirradiated third instar larvae treated similarly.

RESULTS

fs(2)P52 mutant embryos have an increased number of pseudo-yolk nuclei

The components required for the first 13 rounds of nuclear division in the *Drosophila* embryo are maternally provided, and consequently screens for female sterility have identified a number of genes required for the nuclear division cycles of early embryogenesis. In a screen of female sterile mutants to identify genes required for nuclear division cycles during embryogenesis, we identified a strain, *fs(2)P52*, carrying a single pUChsneo insertion (Cooley et al., 1988) in which homozygous females lay fertilised eggs which show an abnormal distribution of nuclei as syncytial blastoderm embryos.

In wild-type embryos, nuclei are initially in the interior of the embryo (Fig. 1A,B), and then migrate to the surface (Fig. 1C), where they undergo 3 more cycles of division (Fig. 1D) before cellularisation in cycle 14 (Fig. 1E). The yolk nuclei seen in the interior of the syncytial blastoderm (Fig. 1E) originate from nuclei that failed to migrate to the cortex during nuclear cycle 8, together with those that fall from the surface between interphase 8 and 9. These yolk nuclei divide twice before undergoing two rounds of endoreplication. In contrast, although embryos from females homozygous for the mutation *fs(2)P52*, appear normal during the early stages of their development (Fig. 1A-C), by cycle 10 the majority have an increased number of yolk-like nuclei (Fig. 1D). As development proceeds, the number of these nuclei increases dramatically (Fig. 1E-F), so that by gastrulation morphogenesis appears extremely disrupted. The internal nuclei in the mutant embryos also differ from wild-type yolk nuclei in their distribution. In wild-type embryos the cortical layer of yolk-free cytoplasm slowly increases in depth during interphase of cycles 10-12 and then more rapidly during interphase of division cycle 13, resulting in the yolk nuclei being restricted to a small region along the anterior-posterior axis of the embryo (Fig. 1E-F). In embryos whose mothers are homozygous for the mutation *fs(2)P52*, the pseudo-yolk nuclei are distributed throughout the interior of the embryo (Fig. 1E-F).

To verify that the insertion of the pUChsneo transposon was responsible for the sterile phenotype of *fs(2)P52*, a biotin labelled P-element probe was hybridised to polytene chromosomes from the *fs(2)P52* mutant stock and a single P element detected at 21B. The chromosome deficiency *Df(2L)TE75*, which deletes the region from 21A1 to 21B4-6, failed to complement the female sterile phenotype of *fs(2)P52*. Thus, the pUChsneo transposon inserted at 21B is likely to cause this female sterile mutation. Secondly, the female sterile phenotype of *fs(2)P52* could be reverted by precise or partial excision of the pUChsneo transposon using the Δ2-3 element. The reversion frequency of 5.4×10^{-2} is similar to that reported for the precise excision of other P-element mutations (Kidwell 1986) (see Materials and Methods). The loss of the P-element from 21B was also shown by Southern blot experiments (see Materials and Methods).

The phenotype of *fs(2)P52* together with the location of the transposon led us to test whether this female sterile mutant is an allele of *throng*, which is also the result of a P-element insertion (Shamanski and Orr-Weaver, personal communication). We found that the female sterile *fs(2)throng*, failed to complement the sterility evident in *fs(2)P52* females. Shamanski and Orr-Weaver found that the number of nuclei in the interior of *throng* embryos from cycles 9-13 begins to increase and that by the cellular blastoderm stage, approximately 80% of *throng* embryos appear abnormal. Most of the mutant embryos fail to survive past germ band extension, with 100% of *throng* embryos phenotypically mutant by 20 hours of development. The mutant embryos had the same number of surface nuclei as wild-type embryos at cellular blastoderm, but twice as many internal nuclei.

throng corresponds to the *Gsl* gene

The inclusion of an origin of replication and the gene conferring ampicillin resistance in the pUChsneo transposon (Steller and Pirotta, 1985), allows the isolation of genomic DNA flanking the insertion in *fs(2)P52* by plasmid rescue (see Materials and

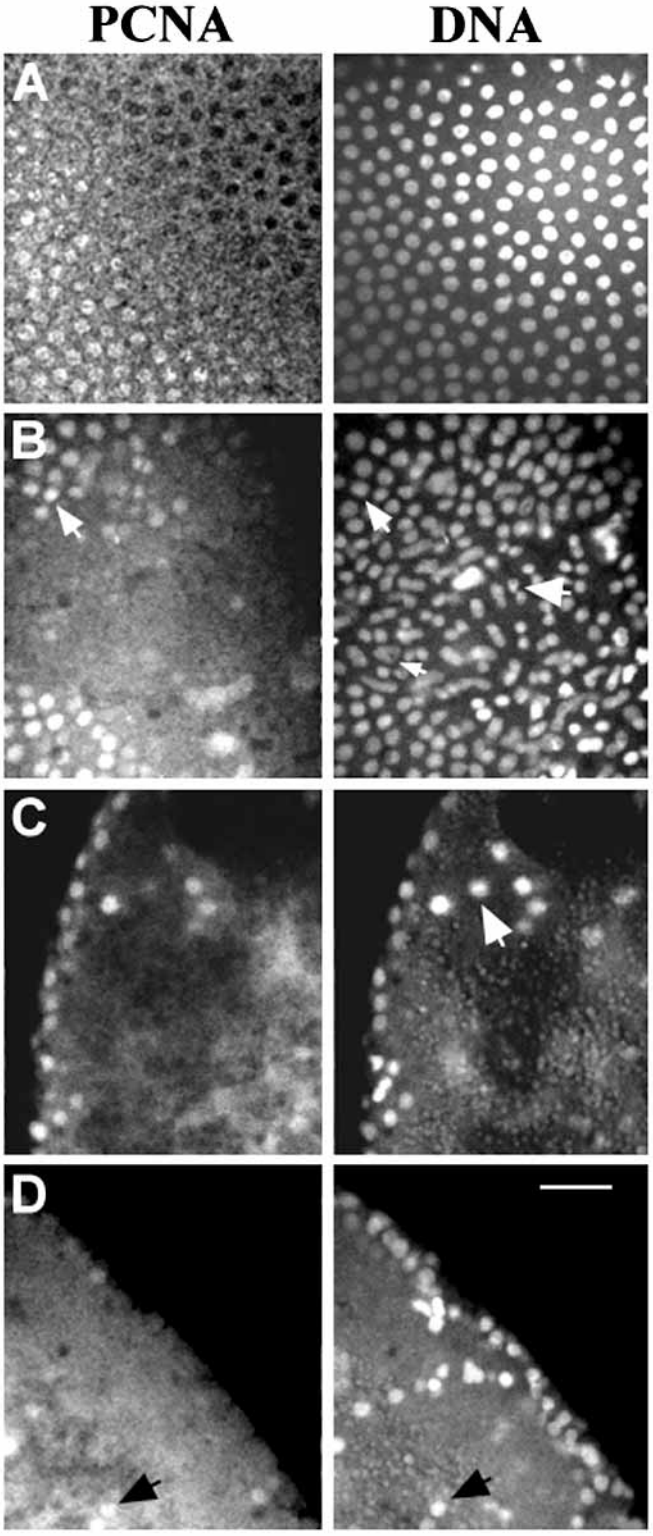

Fig. 5. PCNA distribution in *GsI*^{fsP52}-derived embryos. Wild-type (A) and *GsI*^{fsP52} embryos (B-D) were fixed and incubated with a rabbit polyclonal anti-*Drosophila* PCNA antibody, then with fluorescein-conjugated anti-rabbit IgG, and propidium iodide (see Materials and Methods). (A) The nuclei at the top right of the panel are in anaphase and do not contain the PCNA antigen. As the nuclei proceed through telophase and into interphase (A, bottom left), PCNA becomes localised to the nuclei. (B) A range of mitotic abnormalities frequently associated with *GsI*^{fsP52} embryos of the syncytial blastoderm stage and the distribution of the PCNA antigen in these mitotic figures. Towards the centre of the picture the nuclei are in anaphase, with many of the daughter nuclei remaining linked by chromatin bridges (large arrow). None of these nuclei contain PCNA. The amount of PCNA in individual nuclei appears generally variable, with some staining far more brightly than others, even though their DNA content appears normal (medium arrows). Decondensed nucleus lacking PCNA is shown by the small arrow. (C) Nuclei that have fallen out of the cortex. Initially these cycle in synchrony with the surface nuclei, so that when the surface nuclei contain PCNA, the antigen is also found in the fall-in nuclei. Arrow points to a sub-surface nucleus not showing PCNA staining. (D) When PCNA is absent from the surface nuclei, it is also absent from the nuclei found beneath the surface of the embryo. There are nevertheless exceptions to this general observation (arrows). Bar, 25 μm.

Methods). The flanking genomic DNA isolated in this way was used to screen a library of *Drosophila melanogaster* genomic DNA in lambda DASH. The physical maps of the genomic clones showing the insertion site of the pUChsneo transposon are shown in Fig. 2. The genomic DNA at the insertion site was then used to screen a *Drosophila melanogaster* 0-4 hour embryonic cDNA library (Brown and Kafatos, 1988). Probes λP52B1 and λP52B2 identified the same 6 related clones. These fell into 2 different size categories, the largest of which had an insert of 2.1 kb and is represented by P52c1 in Fig. 2, with the other clones, represented by P52c5, being shorter at the 3′ end and having inserts of 1.5 kb. Physical mapping suggested that the pUChsneo transposon is inserted in the upstream sequences of the gene corresponding to the cDNAs. The analysis also indicated that there is a small intron at the 5′ end of this transcribed region. The insert of the cDNA clone P52c1 was sequenced (Fig. 3) and was found to include a consensus start codon, a poly(A) tail and an ORF that encodes a predicted protein of 399 amino acids, identical to *Drosophila* glutamine synthetase I (GSI) (Caizzi et al., 1990). The sequence of the ends of the inserts of the other class of cDNA clones was also determined and these were found to represent a smaller transcript of *GsI*, resulting from the use of one of the other 2 alternative polyadenylation signals. Sequence analysis of the plasmid rescue constructs described above indicate that the pUChsneo transposon is inserted within the 5′ untranslated sequence of the transcript, 160 bp from the start codon. Furthermore, a 220 bp intron was located 7 bp 5′ of the *GsI* start codon.

We subsequently attempted to rescue the *fs(2)P52* mutation with a germline transformation construct, pw8.P52, that includes the genomic fragment corresponding to the transcribed region, 0.5 kb of upstream sequence and 0.7 kb of genomic DNA downstream of the gene (Fig. 2) inserted into the pw8 vector (Klemenez et al., 1987). We isolated three *Drosophila* lines transformed with the pw8.P52 construct, all of which were able to complement the *fs(2)P52* mutation, confirming that the wild-type gene is included in this genomic fragment. To confirm that the gene responsible for the rescue of the mutant phenotype was the *GsI* gene, the rescue experiment was attempted using a related construct in which the *GsI* open reading frame had been disrupted (see Materials and Methods). We obtained three *Drosophila* lines transformed

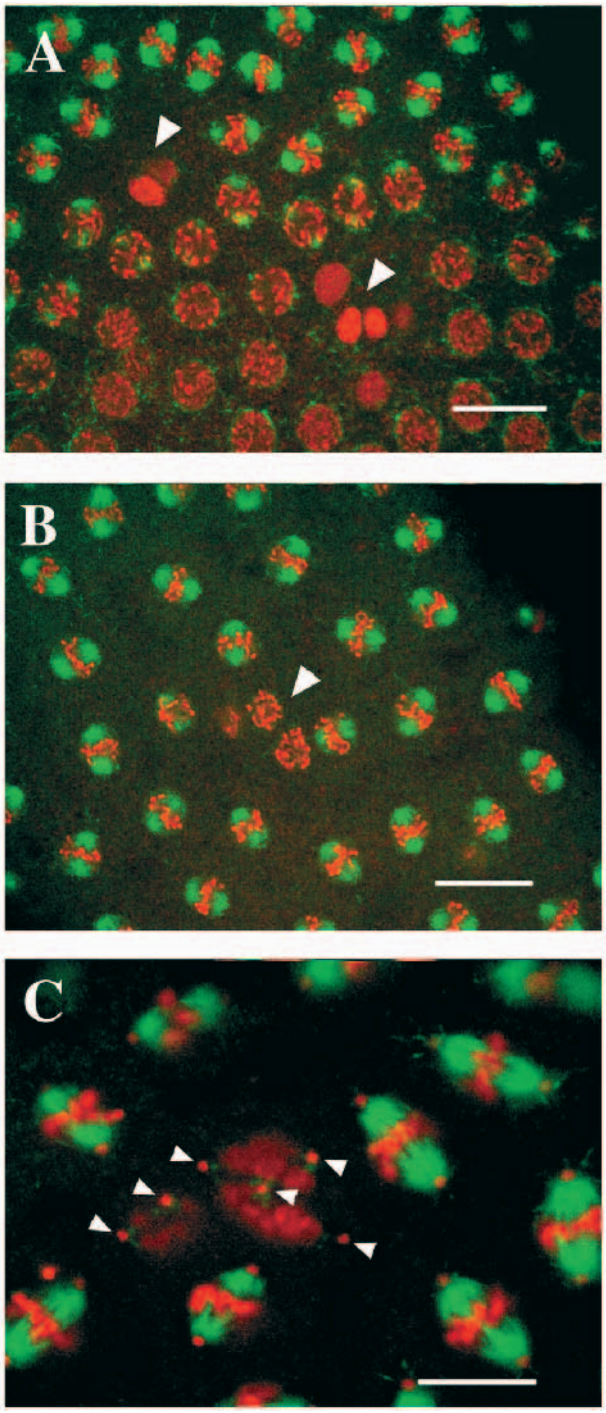

Fig. 6. Asynchronous mitotic progression in *GsI*^{fsP52}-derived syncytial embryos. *GsI*^{fsP52} embryos were fixed and immunostained as described in the legend to Fig. 4. (A) Interphase-like nuclei (arrowheads) surrounded by a gradient of prometaphase to metaphase figures. (B) A cluster of prophase nuclei without associated microtubules (arrowhead) surrounded by metaphase figures. (C) A cluster of abnormal nuclei with decondensed chromatin associated with centrosomes nucleating small asters of microtubules (arrowheads), and surrounded by a field of normal looking metaphase figures. Bars: in A and B, 25 μm; in C, 10 μm.

with this construct, none of which was able to rescue the *fs(2)P52* mutation. Subsequently, we refer to the *fs(2)P52* and *throng* mutations as *GsI*^{fsP52} and *GsI*^{throng}, respectively.

Nuclear fall-out in *GsI*^{fsP52} embryos

To try and gain insight into how mutations in the *GsI* gene can result in maternal effect lethality associated with the throng phenotype, we undertook a more detailed analysis of mutant embryos in order to determine how the abnormalities might arise. To assess the origin of the nuclei in the interior of *GsI*^{fsP52} embryos we examined the mitotic divisions at the time of nuclear migration in blastoderm embryos. In many cases, we observed *GsI*^{fsP52} syncytial blastoderm embryos in which the mitotic divisions appeared to be occurring normally, with the exception of small areas of cortex which lacked nuclei (Fig. 4), but nevertheless had centrosomes (Fig. 4E, arrowheads) and asters (Fig. 4A, arrowhead). Optical sections on lower focal planes suggest that nuclei have fallen into the interior of the embryo (Fig. 4B, arrowhead), leaving behind the microtubular asters with which they were associated. Evidence of abnormal mitoses can be seen in Fig. 4C and D. Although the majority of surface nuclei shown in Fig. 4C are in telophase, a number of the daughter nuclei remain connected by chromatin bridges, that have not fully separated at anaphase (small arrowhead). In this embryo the usual equidistant spacing of the nuclei has also been lost and chromatin bridging between adjacent nuclei is sometimes evident (large arrowhead). A subcortical section of this region (Fig. 4D) reveals a number of nuclei which appear variable in both size and shape. These have probably sunk from the surface of the embryo. Fig. 4E and F show optical sections of an embryo with cortical nuclei in metaphase. Underlying 2 pairs of centrosomes at the cortex (Fig. 4E, arrowheads) is a polyploid nucleus (Fig. 4F, arrowhead) that could have resulted either from a failure to complete a previous mitotic division or the fusion of adjacent telophase nuclei in the previous cycle. Nuclei have previously been described as falling into the interior of the embryo in a number of mutations where the mitotic divisions have failed in some respect, a process that serves to remove any abnormal nuclei from the developing embryo (Minden et al., 1989; Sullivan et al., 1993b; Fogarty et al., 1994; see Discussion).

Nuclei which lose their association with the cortex are unlikely to continue dividing since they no longer have centrosomes associated with them. We wished to determine whether these sub-cortical nuclei continue to prepare for DNA replication by accumulating PCNA. PCNA, a cofactor of DNA polymerase δ is found in the nucleus during interphase, but is cytoplasmic during mitosis (Ng et al., 1990). In wild-type embryos, DNA decondenses as the nuclei enter interphase and PCNA becomes localised to these nuclei (Fig. 5A). In *GsI*^{fsP52} mutant embryos some of the decondensed nuclei have an abnormal morphology and also lack PCNA (Fig. 5B, small arrowhead). These nuclei are no longer cycling in a normal fashion, if at all, suggesting that they have either arrested prior to DNA replication, or are no longer bound by the temporal controls usually regulating the nuclear division cycles. We found that when the surface nuclei contain PCNA, most of the sub-cortical nuclei also contain this antigen, although the levels of PCNA in the internalised nuclei appear rather variable (Fig. 5C). Conversely, when PCNA is not detected in the nuclei at the cortex of the embryo, it also appears absent from nuclei

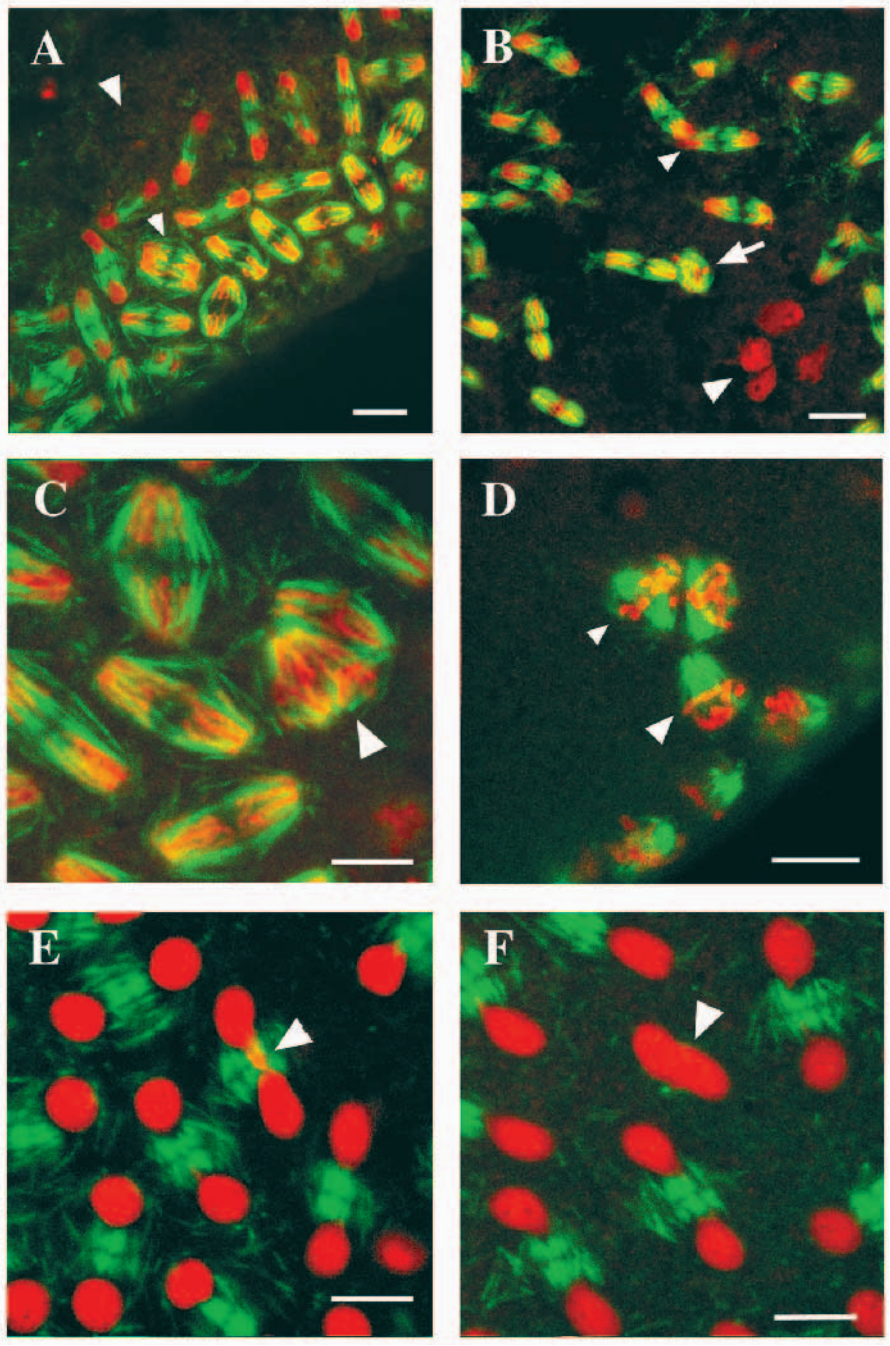

Fig. 7. Abnormal mitotic figures in *GsI*^{fsP52}-derived embryos. *GsI*^{fsP52} embryos were fixed and incubated with YL1/2, a rat monoclonal anti-tubulin antibody, then with fluorescein-conjugated anti-rat IgG, and propidium iodide (see Materials and Methods). In the merged image, DNA is represented in red, with tubulin in green. The figure illustrates examples of the abnormal mitotic spindles evident in *GsI*^{fsP52} syncytial blastoderm embryos, as well as the abnormal telophase phenotype commonly seen in this mutant (see text for details). Bars: in A and B, 25 μm; in D, 20 μm; in C, E and F, 10 μm.

found below the surface (Fig. 5D). There are exceptions to this general observation (arrowheads) and it is likely that, as with the yolk nuclei, the synchrony is eventually lost as the nuclei fall further into the interior of the embryo.

Mitosis is frequently abnormal in *GsI*^{fsP52} embryos

The *GsI*^{fsP52} mutant embryo illustrated in Fig. 4B also shows abnormal patterns of chromatin distribution during mitosis. In many of the anaphase and telophase figures, daughter nuclei remain linked by bridging strands of chromatin (Fig. 4B, large arrowhead). We therefore made a closer examination of mitosis in *GsI*^{fsP52} embryos at each stage of the division cycle. Mitotic waves progress across mutant embryos as in wild type, but the synchrony of divisions appears to be lost in the division of individual nuclei within any given field. Thus a few abnormal mitotic figures are evident in a field of otherwise normal nuclei. The abnormalities frequently seen are of nuclei at an inappropriate stage of the division cycle by comparison with their neighbours. The DNA of some nuclei appeared unable to condense properly prior to division (Fig. 6A, arrowheads). Examples are also seen in which chromosome condensation occurs normally, but the mitotic spindle fails to form (Fig. 6B, arrowhead). Such abnormal nuclei are often still associated with centrosomes that are able to nucleate small asters of microtubules rather than bipolar spindles (Fig. 6C).

In addition to asynchrony of mitosis, we also observed

abnormal mitotic figures within a synchronous field of dividing nuclei (Fig. 7). The surface of the embryo in Fig. 7A shows an area lacking nuclei (large arrowhead), while one of the mitotic figures appears polyploid and has broad poles resulting in a more barrel-shaped spindle (small arrowhead). In Fig. 7B there are further examples of abnormal spindle structures (arrow); an anaphase figure with a shared pole (small arrowhead); and of non-dividing nuclei not associated with microtubules (large arrowhead). The regular spacing between adjacent spindles is often disrupted (Fig. 7C), and the juxtaposed spindles can have broad poles (arrowhead). Hemispindles (Fig. 7D, large arrowhead) and tripolar structures (small arrowhead) can also be observed in sub-cortical regions, although this phenotype is less common. Bridging between daughter nuclei and lagging chromosomes are frequently evident in telophase figures, although midbodies appear normal (Fig. 7E, arrowhead). In many instances daughter nuclei appear ultimately to fuse with each other to result in polyploid nuclei (Fig. 7F, arrowhead).

GsI^{fsP52} confers hypersensitivity to the O_2^--generating agent, paraquat and to X-irradiation

In addition to affecting the levels of glutamine, it is possible that the accumulation of glutamic acid and ammonium ion donors resulting from a reduction in GSI activity could have other metabolic affects, such as the perturbation of urate and glutathione metabolism. As glutathione peroxidase is one means of detoxifying reactive oxygen intermediates, we decided to test the susceptibility of *GsI*^{fsP52} to the DNA damaging affects of X-rays and paraquat, an agent which induces free radical formation (see Materials and Methods). Flies with a mutation in the *ry* gene, which encodes xanthine dehydrogenase (Glassman and Mitchell, 1959), have previously been demonstrated to show an increased sensitivity to these agents as a result of the role of this enzyme in urate production, a known anti-oxidant (Hilliker et al., 1992). We found that males homozygous for the *GsI*^{fsP52} mutation are more sensitive to paraquat (LD_{50}=6 mM) than Oregon-R males (LD_{50}=20.5 mM), and showed a similar degree of paraquat sensitivity to males mutant for the *ry* gene (LD_{50}=5.5 mM) (Fig. 8A). Upon exposure of third instar larvae to 2,000 rads of X-irradiation, 69% of Oregon-R larvae developed into adult flies, whereas only 38% of *ry* and 50% of *GsI*^{fsP52}appeared as adults, when compared to the survival rate in the absence of irradiation (Fig. 8B).

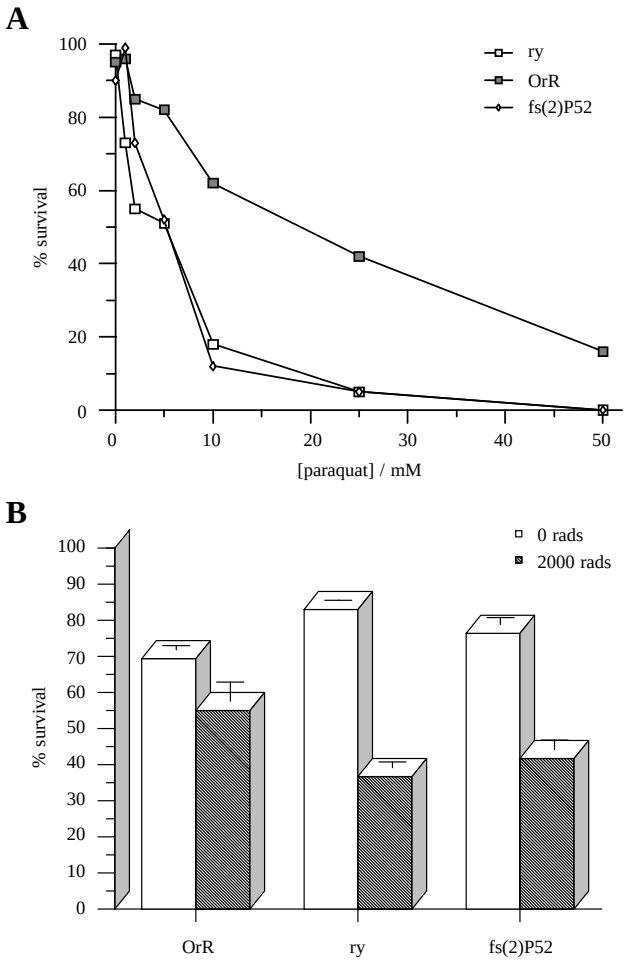

Fig. 8. *GsI*^{fsP52} confers hypersensitivity to paraquat and X-radiation. (A) Adult males were exposed to paraquat as described in Materials and Methods. A minimum of 100 adults of each genotype were assessed for survival after treatment for 48 hours. (B) Third instar larvae were exposed to 0 rads or 2,000 rads of X-radiation (see Materials and Methods). The survival rate was calculated as the number of larvae eclosing as adults. A minimum of 100 larvae of each genotype were tested in each of 3 independent experiments and the results given as mean values with standard deviations.

DISCUSSION

In this paper, we have characterised a female sterile mutation, *fs(2)*P52, which we have discovered is an allele of *throng*. Molecular cloning and sequencing has shown this gene to encode glutamine synthetase I. This is one of two isoforms of the enzyme, both of which catalyse the ATP-dependent production of glutamine from glutamate and ammonia (Meister, 1980). GSII is present mainly in the thorax of the fly, whereas GSI is present in the head, thorax, abdomen and reproductive organs (Caggese et al., 1992). GSI is reported to be the mitochondrial form of the enzyme, whereas GSII is cytosolic (Caizzi et al., 1990), although this has not been examined in embryos, where there is almost no GSII present and GSI is contributed by the mother. A number of lethal alleles of *GsI* have been described in which GSI activity is absent altogether (Caggese et al., 1988), demonstrating that some GSI activity is required for *Drosophila* survival. Caggese et al. (1992) have also discovered a female sterile mutation of *GsI*, which resulted in a reduction of GSI activity, but the phenotypes of the mutant embryos were not examined. GSII activity was able to substitute for GSI activity in the head of the fly, but remained absent from the ovaries and embryos from these females. It therefore appears that, although GSII can functionally substitute for GSI to some degree, this is not the case for the maternal provision of GSI to the embryo.

We describe the dramatic consequences of the hypomorphic mutation *GsI*^{fsP52} upon the nuclear division cycles in the syncytial embryo. The embryos from homozygous mutant females show a number of irregularities following the migration of the nuclei to the cortex. Most notable, is the variation in the size and shape of the nuclei. This suggests that nuclear fusion events, division failure or other division abnor-

malities have taken place. Many of the embryos have patches of nuclei differing in their density and/or stage in the division cycle from that of their neighbours. In some regions of the cortex nuclei are absent altogether, although centrosomes are still present. The abnormal nuclei appear to fall from the cortex into the interior of the embryo, with the number of these internal nuclei increasing during the development of the embryo. The internal nuclei evident in *GsI*^{fsP52} embryos may result from failure to migrate to the cortex during cycle 10, or from falling out of the cortex in subsequent cycles. However, the similarity of the *GsI*^{fsP52} phenotype to other mutants in which internal nuclei are attributed to nuclear fallout following abnormal divisions and the appearance of abnormal mitotic figures at the surface of *GsI*^{fsP52} embryos (Fig. 4C, arrowheads) favours nuclear fallout being responsible for the internal nuclei in this mutant. This appears to be a mechanism used in the syncytial embryo to eliminate abnormal nuclei. In embryos mutant for *scrambled*, *nuclear-fallout* and *sponge*, actin dynamics are defective and cortical nuclei undergo abnormal divisions and ultimately sink into the interior of the embryo (Sullivan et al., 1993a; Postner et al., 1992). In embryos from females homozygous for the mutation, *daughterless-abo-like (dal)*, centrosomes appear to fail to separate during interphase, resulting in a non-functional spindle and nuclear division failure. Additionally, abnormal spindles form using a centrosome from a neighbouring nucleus so that nuclei share a common spindle pole. The abnormal spindles result in telophase fusions and other mitotic abnormalities, with affected nuclei falling out of the cortex into the interior of the embryo. In the *Drosophila* maternal-effect mutation *grapes* (Fogarty et al., 1994) failure to form midbodies during anaphase and telophase of nuclear cycle 12 results in the collision of sister telophase nuclei to produce a tetraploid nucleus that arrests in metaphase in division cycle 13. After some considerable time, adjacent polyploid nuclei aggregate into grape-like clusters, some of which fall into the interior of the embryo.

A striking characteristic of the *GsI*^{fsP52} embryonic phenotype is the appearance of nuclei delayed in nuclear division cycle progression. The majority of the abnormal nuclei are either at anaphase/telophase, with many of these showing chromatin bridging, or have a decondensed interphase-like morphology. This suggests that the nuclear division cycle may be arresting at these points prior to the nuclei being discarded from the cortex. In support of this Sullivan et al., (1993) reported that a delay in anaphase in embryos bearing an abnormally long and rearranged second chromosome, leads to severely abnormal divisions or nuclei completely failing to divide. These nuclei also appear to fall from the cortex into the interior of the embryo. Many of the phenotypic characteristics of *GsI*^{fsP52} mutant embryos might be explained by nuclei losing their association with centrosomes as a result of their inability to complete anaphase, as microtubule asters emanating from the centrosomes are responsible for maintaining the equidistant spacing of the nuclei at the cortex.

Why should a lack of glutamine synthetase lead to such dramatic effects upon cell cycle progression? Is it a consequence of having insufficient glutamine, or the accumulation of glutamic acid and ammonium donors? Glutamine plays a central metabolic role, providing nitrogen for the synthesis of amino acids such as tryptophan, histidine and asparagine. These metabolites are clearly essential for the high demands upon protein synthesis during oogenesis and embryogenesis. During early embryonic development many of the proteins required by the embryo are provided by the mother. The female also supplies the embryo with a number of mRNAs, which are translated during the early division cycles in the embryo. The need for protein synthesis during syncytial development was demonstrated by Zalokar and Erk (1976), who treated embryos with the protein synthesis inhibitor, cycloheximide and found that embryogenesis was arrested in the subsequent interphase. This inhibition may function by interfering with the turnover of cyclins (Edgar et al., 1994) which becomes apparent once the nuclei have migrated to the cortex, a time at which the mutant phenotype of *GsI*^{fsP52} is seen. At the very least, a reduction in amino acid availability would delay cycle progression to accommodate the reduced efficiency of protein synthesis.

Glutamine is also essential for the biosynthesis of purines and pyrimidines. The demands of the embryo for purines and pyrimidines are exceptionally high to maintain a level of DNA replication that will ultimately generate of the order of 10^4 diploid nuclei, utilising maternally provided precursors. To accommodate such demands the enzymes involved in the pathways for de novo synthesis of these DNA precursors are enriched in the embryo. Glutamine is required to provide nitrogen for the synthesis of these compounds. Therefore, it is likely that any reduction in the levels of glutamine, particularly in the embryo where demands are so high, might drastically reduce the availability of purines and pyrimidines for DNA synthesis. This could result in delays in S-phase or incomplete DNA replication and failure of subsequent mitotic divisions. At other stages of the *Drosophila* life cycle, demands for purine and pyrimidine synthesis are not so extreme. Furthermore, unlike later developmental stages, the checkpoints that monitor the completion of DNA replication are not functional in the syncytial embryo (Raff and Glover, 1988), thus allowing incomplete DNA replication to lead to mitotic abnormalities. Some DNA replication does take place in the abnormal nuclei seen in *GsI*^{fsP52} embryos, as judged by the incorporation of BrdU (data not shown) and by the nuclear distribution of PCNA. However, the pattern of association of PCNA with the nuclei in mutant embryos suggests this aspect of cell cycle progression can lose synchrony in some nuclei. It may be that DNA replication is not completed successfully in the available time period or that the embryos fail to correct errors in the DNA prior to the next cycle, so that mitotic abnormalities occur.

Although a depletion in the levels of glutamine potentially affects DNA replication in general, experiments in which the *GsI*^{fsP52} mutants were exposed to the radical generating agent, paraquat, and to X-rays, suggest that the hypomorphic mutation *GsI*^{fsP52} may also have a significant effect on the response to oxidative stress, with subsequent effects upon cell cycle progression as a consequence of DNA damage. We propose that this may be the result of the reduction in GSI activity altering the levels of urate or glutathione, both of which are known anti-oxidants.

The consequences of a deficiency for this essential metabolic enzyme thus appear to be highly pleiotropic. As the major biosynthetic activity of the embryo at this developmental stage

is the nuclear division cycle, it is perhaps not surprising that this process suffers the major consequence. The very rapidity of these cycles places a huge metabolic load upon the cell, and the lack of several checkpoint controls allow an accumulation of cell cycle defects, resulting in the repeated loss of nuclei from the cortex of the embryo as they fall into the interior.

This work was supported by grants from the Cancer Research Campaign. L.M.F. was the holder of a Research Studentship from the Science and Engineering Research Council (SERC). We thank Fay Shamanski and Terry Orr-Weaver for communicating to us their unpublished data on *throng*, and providing us with this mutant. We acknowledge helpful discussion from many of our colleagues during the course of this work, and the helpful comments of Emma Warbrick on the manuscript.

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(Received 8 May 1996 - Accepted 22 July 1996)